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# Endocytic Pathway Alterations in Human Hippocampus after Global Ischemia and the Influence of *APOE* Genotype

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**Apolipoprotein  $\epsilon 4$  (apoE, protein; *APOE*, gene) allele is the most important genetic risk factor for development of Alzheimer's disease and is also associated with poor outcome after brain injury. Although the mechanisms underlying this susceptibility are currently unknown, recent experimental evidence suggests that *APOE* genotype may influence activity in the endocytic pathway of neurons. This study determined whether alterations in the endocytic pathway occurred in medial temporal lobe sections after brain injury because of cardiorespiratory arrest and whether these alterations were influenced by *APOE* genotype. Antibodies to two proteins involved in endocytosis, rabaptin-5 and rab4, were used as markers of endocytic pathway activity. Alterations in immunoreactivity were examined in medial temporal lobe sections in the postmortem brain of patients who experienced an episode of global ischemia and in controls. After global ischemia there was a marked increase in immunoreactivity of both endocytic markers, rabaptin-5 and rab4, in neurons, and to a lesser extent in glia compared to controls. Furthermore, possession of an *APOE*  $\epsilon 4$  allele was associated with specific alterations in the endocytic pathway. After global ischemia, there was no influence of *APOE* genotype on the extent of rabaptin-5 immunoreactivity. However, there was a statistically significant influence of *APOE* genotype on the extent of rab4 immunoreactivity in response to global ischemia. These results indicate marked alterations in the endocytic pathway after global ischemia that are dependent on**

***APOE* genotype. This may underlie the important influence of *APOE* genotype on brain injury and disease. (Am J Pathol 2003, 162:273–281)**

Endocytosis is an intracellular trafficking process whereby macromolecules are transported from the plasma membrane to the cellular cytosol in a series of intracellular compartments or vesicles. In neurons, the endocytic pathway is involved in processes common to various cell types such as the uptake of nutrients and trophic factors. In addition, neuronal endocytosis is intimately associated with events after neurotransmitter release including the internalization, degradation, and recycling of plasma membrane receptors and their associated ligands.

Early and late endosomes and lysosomes comprise the group of intracellular membrane-bound compartments also known as the central vacuolar system. The early endosome receives receptor/ligand complexes from internalized clathrin-coated pits and is the first major sorting station in the pathway.<sup>1</sup> From the early endosome, molecules that have been sequestered by neurons have three major fates: recycling back to the plasma membrane, particularly receptors; transport to intracellular membranous organelles for further sorting and distribution; and degradation by the lysosomal proteolytic enzymes.<sup>2–4</sup>

The various pathways and stages of endosomal trafficking are regulated by numerous proteins, with the Rab enzymes particularly prominent.<sup>1,4–7</sup> The Rab proteins are a group of GTPases that localize to specific components of the endocytic machinery and regulate distinct stages of endocytic trafficking. Three proteins whose actions have been well documented are rab4, rab5, and rab7. Rab4 associates with recycling endosomes and is required for efficient transport of molecules back to the plasma membrane.<sup>8</sup> Rab5, in contrast, localizes to early endosomes and is involved in the internalization stage of endocytosis.<sup>9</sup> Rab7 is essential for the trafficking of mol-

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ecules from early to late endosomes.<sup>10</sup> In addition, the role of rabaptin-5, an effector of rab5 that is recruited to early endosomes during periods of endocytic activation, has also been documented.<sup>7</sup>

The endocytic pathway is a key point of convergence of several proteins implicated in Alzheimer's disease (AD) pathophysiology, such as amyloid precursor protein, amyloid- $\beta$  (A $\beta$ ), and apolipoprotein E (apoE, protein; *APOE*, gene). In view of this, there has been increasing interest in the role of endocytic alterations in neuronal dysfunction. Observations in AD postmortem brain have indicated that there is endocytic activation and up-regulation of lysosomal activity.<sup>11,12</sup> Using an antibody to rab5, it was shown that there is an increase in the number of rab5 immunoreactive early endosomes, and a marked enlargement of these endosomes in neocortical pyramidal neurons. The size and number of early endosomes is believed to be proportional to the rate of endocytic uptake,<sup>13–15</sup> and therefore, the observations above would suggest an increase in endocytic uptake. Furthermore, these alterations precede the appearance of AD pathology, such as amyloid deposits.<sup>12</sup> It has also been shown that rabaptin-5, a marker of internalization, interacts with neuronal growth-associated protein.<sup>16</sup> Growth-associated protein plays an important role in neuronal plasticity after injury and therefore endosomal alterations may also be involved in the plastic response to neuronal insult.

ApoE is a polymorphic protein that exists as three major isoforms; E2, E3, and E4, encoded by the alleles  $\epsilon$ 2,  $\epsilon$ 3, and  $\epsilon$ 4. Possession of one or more copies of the  $\epsilon$ 4 allele is a major risk factor for late-onset familial and sporadic AD<sup>17</sup> and for poor outcome after head injury.<sup>18</sup> Patients with the *APOE*  $\epsilon$ 3 genotype show a significantly higher survival rate and increased chance of a favorable neurological outcome compared to non-*APOE*  $\epsilon$ 3 individuals after cardiopulmonary resuscitation.<sup>19</sup> Studies using animal models of cardiac arrest have provided evidence of an *APOE* genotype influence. ApoE-deficient mice have increased neuronal damage after global ischemia.<sup>20</sup> *APOE*  $\epsilon$ 4 mice have more extensive neuronal damage and apoE immunoreactivity after global ischemia compared to *APOE*  $\epsilon$ 3 mice.<sup>21</sup> The mechanisms underlying this susceptibility remain unclear and as yet alterations in the endocytic pathway have not been determined.

This study tested the hypothesis that alterations in the endocytic pathway occur after brain injury and that this is *APOE* genotype-dependent. Markers of the endocytic pathway were examined in postmortem brain from patients who experienced an episode of global ischemia because of cardiac arrest and were compared to neuropathologically normal controls. To detect changes in endocytic pathway activity we used antibodies to two proteins involved in different stages of endocytosis, rabaptin-5 and rab4. Rabaptin-5 is a cytoplasmic effector protein recruited to the membrane of early endosomes during endocytosis. Rab4 is a protein that localizes to the membrane of endosomes being transported back to the cellular plasma membrane.

## Materials and Methods

### Postmortem Human Brain Tissue

The control and global ischemia groups were matched for age and male:female ratio. Archival paraffin-embedded blocks of medial temporal lobe were selected from 43 patients who died after an episode of global ischemia because of cardiorespiratory arrest (27 males, 16 females; mean age, 50 years). The survival period after the initial episode of global ischemia ranged from 11 hours to 3 months. Thirty-eight control patients (25 males, 13 females; mean age, 51 years) without clinical or neuropathological evidence of neurological or psychiatric impairment or cardiovascular disease, were used in this experiment. *APOE* genotyping was determined from the paraffin-embedded brain tissue as previously described using the polymerase chain reaction/restriction enzyme analysis technique.<sup>22</sup> The success rate for the determination of *APOE* genotype was high; *APOE* genotype was undetermined in only three control patients. Consequently, 35 controls were used when analyzing the influence of *APOE* genotype. The frequency of the *APOE*  $\epsilon$ 4 allele was similar in the control and global ischemia groups. In the control group, 24 individuals were without an  $\epsilon$ 4 allele (mean age, 52 years) and 11 individuals had at least one  $\epsilon$ 4 allele (mean age, 49 years); in the global ischemia group, 28 cases were without an  $\epsilon$ 4 allele (mean age, 52 years) and 15 cases had an  $\epsilon$ 4 allele (mean age, 49 years). This study was approved by the ethics committee of the Southern General Hospital.

### Rabaptin-5 and Rab4 Immunohistochemistry

Paraffin-embedded sections were dewaxed in an oven at 60°C for 40 minutes, and in histoclear (2  $\times$  10 minutes) followed by dehydration in absolute alcohol (2  $\times$  10 minutes). Endogenous peroxidase was eliminated by incubating the sections with 0.5% hydrogen peroxide for 30 minutes followed by washes in running water for 40 minutes and phosphate-buffered saline (PBS) (2  $\times$  5 minutes). Nonspecific binding sites were blocked with 10% normal serum (normal horse serum for rabaptin-5 sections; normal goat serum for rab4 sections, Vector Laboratories, Peterborough, UK) and 0.5% bovine serum albumin in PBS for 1 hour then incubated with primary antibody [rabaptin-5, 1:50, goat polyclonal (Santa Cruz Biotechnology, Wiltshire, UK); rab4, 1:50, rabbit polyclonal (Santa Cruz)] in blocking solution overnight at 4°C. After washing in PBS (2  $\times$  5 minutes) sections were incubated with biotinylated secondary antibody in PBS (anti-goat IgG, 1:100 for rabaptin-5 sections; anti-rabbit IgG, 1:100 for rab4 sections (Vector Laboratories)) for 1 hour. Sections were then washed in PBS (2  $\times$  5 minutes) and then processed with a Vectastain ABC Elite kit. Color was developed using a 3'3'-diaminobenzidine tetrahydrochloride kit (Vector Laboratories). Sections were then dehydrated in a series of alcohols and mounted on glass slides. Controls for the specificity of immunostaining included omission of the primary antibody. Positive controls included hippocampal sections from AD brain.

### *Semiquantification of Rabaptin-5 and Rab4 Immunoreactivity*

Semiquantitative assessment of rabaptin-5 and rab4 immunoreactivity within the hippocampus and neocortex was performed at  $\times 200$  magnification. The cases were coded and the investigator was blind to their identity. The degree of neuronal immunoreactivity was classified using a scoring system as follows: 0, no neuronal staining; 1,  $<35\%$  neurons stained; 2, 35 to 70% neurons stained; 3,  $>70\%$  neurons stained. Glial immunoreactivity was classified similarly. Ischemic neuronal damage in hematoxylin and eosin-stained sections had previously been semiquantified in these cases using a similar assessment to that used above for rabaptin-5 and rab4 immunoreactivity.<sup>23</sup> An average degree of rabaptin-5 and rab4 immunoreactivity and ischemic neuronal damage for each case was calculated from the sum of the degree of neuronal/glial immunoreactivity or ischemic neuronal damage in CA1 + CA2 + CA3/CA4 + dentate fascia + neocortex  $\div$  5.

### *Statistics*

Mann-Whitney *U*-test for nonparametric data were used to assess statistical significance of differences in the degree of neuronal or glial rabaptin-5 and rab4 immunoreactivity between control and global ischemia groups. The Spearman rank correlation coefficient quantified the association of degree of neuronal rabaptin-5 or rab4 immunoreactivity with ischemic neuronal damage and with patient survival time after global ischemia. Spearman rank correlation analysis was also used to assess the association between neuronal rabaptin-5 and rab4 immunoreactivity after global ischemia. The association of *APOE* genotype with endocytosis after global ischemia was determined for rabaptin-5 and rab4 immunoreactivity, two-way analysis of variance tested for an interaction between the effects of presence of an *APOE*  $\epsilon 4$  allele and of an episode of global ischemia. Use of two-way analysis of variance assumes a normal distribution of data. Assumption of normality was reasonable when the average response per subject was calculated (Kolmogorov-Smirnov test,  $P > 0.05$ ).

## *Results*

### *Rabaptin-5 Immunoreactivity*

Rabaptin-5 immunoreactivity was markedly increased in neurons (Figure 1) and to a lesser extent in glia after global ischemia. In controls, there was faint staining of neurons and glia. In the hippocampus, neuronal staining was predominantly confined to the pyramidal cell layer of CA1, CA2, CA3, and CA4 and the granule cell layer of the dentate gyrus. However, a diffuse pattern of staining could also be seen in the molecular layer of the hippocampus in some global ischemia cases. Glial staining was more widespread in nature with a less laminar pattern than the neuronal staining. Glial staining was primar-

ily astrocytic, although reactivity was also observed in cells with the characteristic morphology of microglia. Neuronal rabaptin-5 immunostaining was characteristically granular in nature in the cytoplasm of cell bodies and neurites, indicative of its endosomal location. After global ischemia, there was dense staining of rabaptin-5 adjacent to the plasma membrane (Figure 1c), whereas in controls, when present, rabaptin-5 staining was more widely dispersed within the cytosol (Figure 1a).

Semiquantification of the degree of rabaptin-5 immunoreactivity revealed significant increases in neuronal rabaptin-5 in CA1 ( $P < 0.0005$ ), CA2 ( $P < 0.0005$ ), and CA3/4 ( $P < 0.0005$ ) of the hippocampus and neocortex ( $P < 0.05$ ) after global ischemia compared to controls (Figure 2a). There was also an increase observed in the dentate gyrus, although this was not statistically significant. The majority of global ischemia cases showed moderate to maximal neuronal rabaptin-5 immunostaining (semiquantitative scores of 2 and 3, respectively). In contrast, in most of the controls only minimal staining (semiquantitative score of 1) was evident.

Glial rabaptin-5 immunostaining was detected both in controls and after global ischemia. However, the degree of immunoreactivity was less than that observed in neurons. The majority of controls had minimal or no glial rabaptin-5 staining present and after global ischemia only seven cases showed a moderate or greater degree of staining. Only in CA3/4 of the hippocampus was there a significant increase ( $P < 0.05$ ) in glial rabaptin-5 after global ischemia compared to controls (Figure 2b).

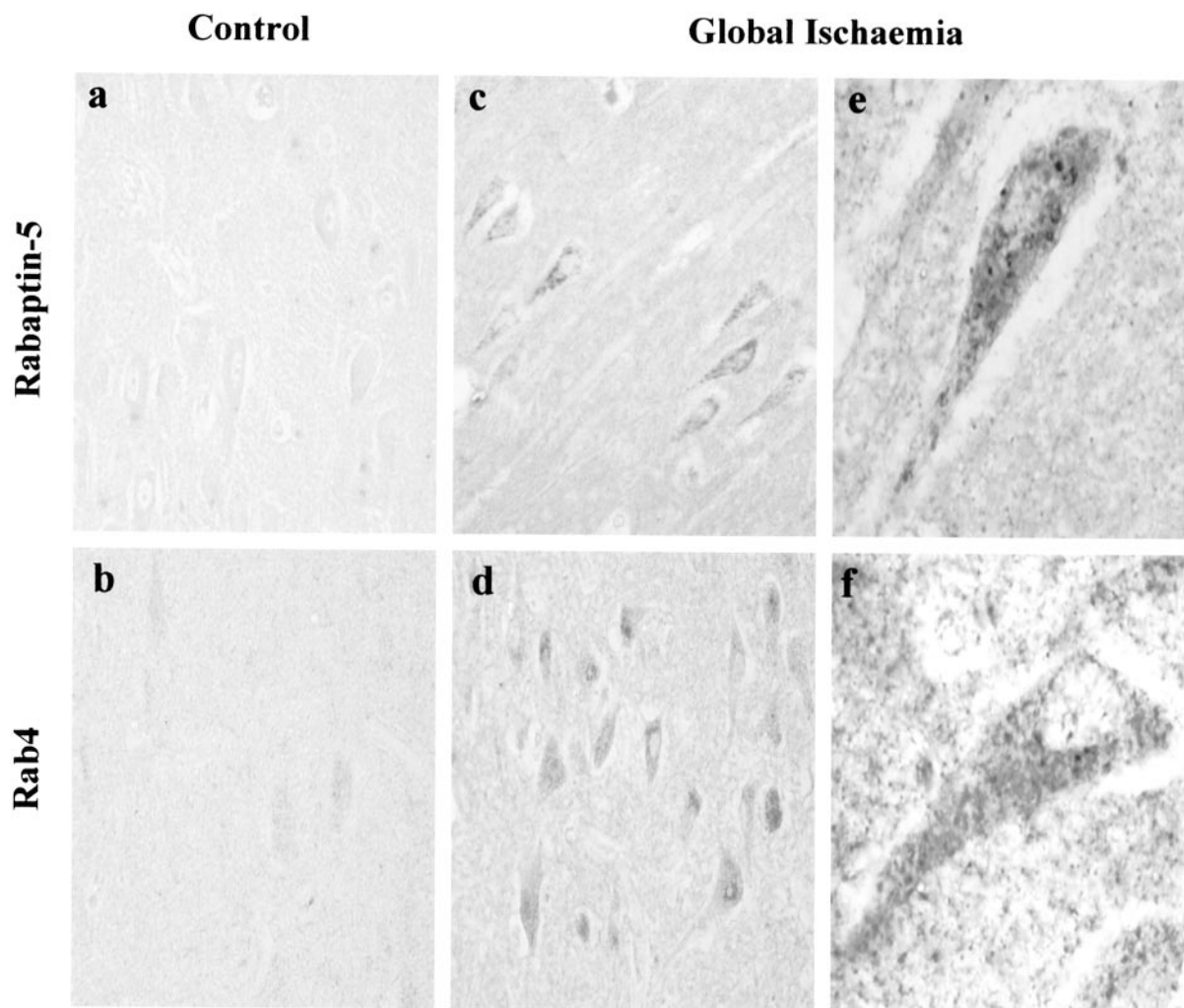
After global ischemia, rabaptin-5 immunoreactivity was observed in nonischemic neurons and in those displaying the characteristic morphological features of ischemic damage. However, there was only a small correlation ( $P = 0.02$ ) between the amount of neuronal rabaptin-5 immunoreactivity and the extent of neuronal damage. There was no association between the level of rabaptin-5 immunoreactivity and neuronal damage in controls. Furthermore, there was no association between the level of neuronal rabaptin-5 immunoreactivity and survival time of the patients who had experienced an episode of global ischemia (data not shown).

### *Rab4 Immunoreactivity*

Akin to the pattern of rabaptin-5 immunoreactivity, rab4 was also detected in neurons (Figure 1) and glia. However, rab4 immunostaining showed greater intensity in glia than rabaptin-5. Intraneuronal rab4 staining was primarily restricted to the pyramidal cell layer of the hippocampus and granule cell layer of the dentate gyrus and had a granular nature in the cytoplasm of cell bodies and neurites. Glial staining was predominantly astrocytic and in some cases after global ischemia intensely stained reactive astrocytes were observed. Furthermore, in adjacent sections stained with rab4 or rabaptin-5 it was evident that identical regions and cells were stained with both antibodies after global ischemia.

Rab4 immunoreactivity was observed in neurons in global ischemia and in control cases. Semiquantification



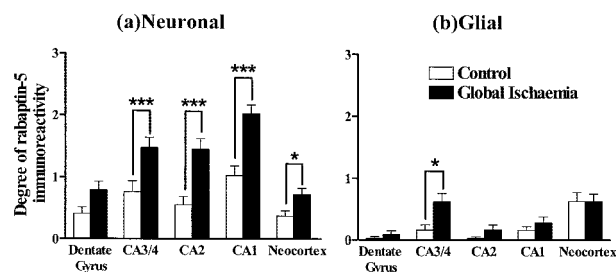


**Figure 1.** Illustrative examples of neuronal rabaptin-5 (a, c, e) and rab4 (b, d, f) immunoreactivity in a patient who experienced an episode of global ischemia (c-f) and a control patient (a, b). In the control patient, there is only faint staining of neuronal cell bodies and processes. In contrast, after global ischemia, there is intense rabaptin-5 and rab4 immunoreactivity in the cytoplasm of neurons. All photomicrographs show CA1 of the hippocampus. Original magnifications: ×400 (a-d); ×1000 (e, f).

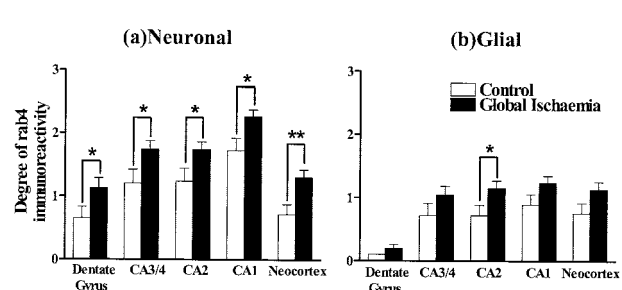
of the degree of neuronal rab4 immunoreactivity revealed significant increases in CA1 ( $P < 0.05$ ), CA2 ( $P < 0.05$ ), CA3/4 ( $P < 0.05$ ), and the dentate gyrus ( $P < 0.05$ ) of the hippocampus and in neocortex ( $P < 0.005$ ) after global ischemia compared to controls (Figure 3a). The majority

of global ischemia cases showed a moderate or greater degree of neuronal rab4 staining whereas, in control tissue, a minimal degree of staining was observed.

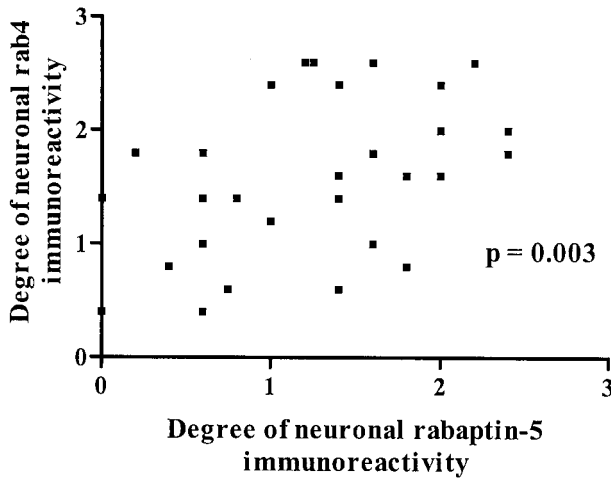
Glial rab4 immunoreactivity exhibited a similar pattern of staining to rabaptin-5; only in one region measured,



**Figure 2.** Semi-quantitative assessment of the degree of neuronal (a) and glial (b) rabaptin-5 immunoreactivity in temporal lobe. There was a significant increase in the level of neuronal rabaptin-5 immunoreactivity after global ischemia compared with controls in all regions examined except the dentate gyrus. Only in CA3/4 was there a significant change in the degree of glial rabaptin-5 immunoreactivity after global ischemia. \*,  $P < 0.05$ ; \*\*\*,  $P < 0.0005$  using Mann-Whitney. Histograms are mean  $\pm$  SEM.



**Figure 3.** Semi-quantitative assessment of the degree of neuronal (a) and glial (b) rab4 immunoreactivity in temporal lobe. There was a significant increase in neuronal rab4 immunoreactivity after global ischemia in all regions examined. Only in CA2 of the hippocampus was there a significant increase in glial rab4 immunoreactivity after global ischemia. \*,  $P < 0.05$ ; \*\*,  $P < 0.005$  using Mann-Whitney. Histograms are mean  $\pm$  SEM.



**Figure 4.** Association between the degree of neuronal rabaptin-5 and rab4 immunoreactivity after global ischemia. There was a positive association between the degree of rabaptin-5 and rab4 immunoreactivity after global ischemia. ( $P = 0.003$  using Spearman's rank correlation analysis) Note: 43 data points were plotted, however, several points are superimposed therefore fewer data points are visible.

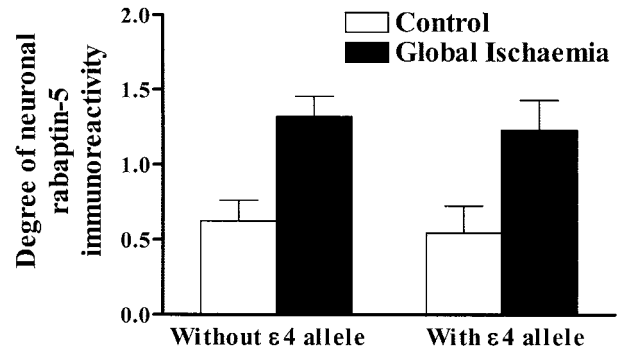
CA2 ( $P < 0.05$ ), was there a significant increase after global ischemia (Figure 3b). However, it was observed that in both controls and global ischemia cases the degree of glial rab4 staining was greater than rabaptin-5. After global ischemia, the majority of cases had minimal or moderate levels of staining, and in controls, a minimal level was predominantly detected.

It was noted that, after global ischemia the degree of neuronal rab4 and rabaptin-5 immunoreactivity was similar although in controls, rab4 displayed a greater level of staining than rabaptin-5. Correlation analysis revealed a moderate association between neuronal rab4 and rabaptin-5 immunoreactivity after global ischemia ( $P = 0.003$ ) (Figure 4).

As observed with rabaptin-5, rab4 immunoreactivity was observed in both nonischemic and ischemic neurons after global ischemia. However, there was no association between the degree of neuronal rab4 immunoreactivity and ischemic neuronal damage after global ischemia. In addition, there was no correlation between neuronal rab4 immunoreactivity and survival time of global ischemia patients.

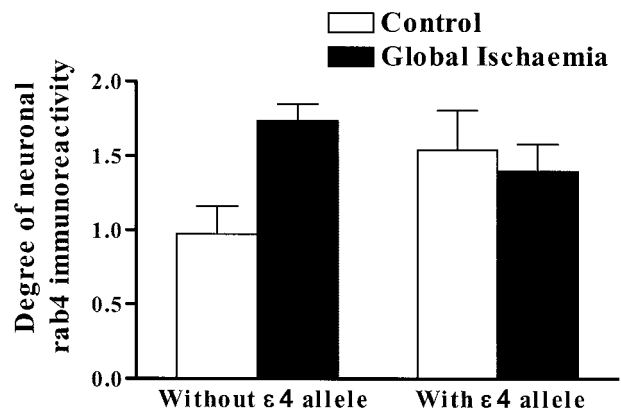
#### Association of APOE Genotype with Changes in Degree of Neuronal Rabaptin-5 and Rab4 Immunoreactivity after Global Ischemia (Figures 5 and 6)

We tested the hypothesis that APOE genotype may be associated with changes in the degree of neuronal rabaptin-5 and rab4 immunoreactivity. The cases were separated into those that contained at least one APOE  $\epsilon 4$  allele and those without an  $\epsilon 4$  allele. The number of individuals homozygous for the APOE  $\epsilon 4$  allele was too low to evaluate whether this genotype was associated with the parameters measured (two controls and two global ischemia cases were  $\epsilon 4$  homozygotes). Likewise,



**Figure 5.** The average degree of neuronal rabaptin-5 immunoreactivity in temporal lobe in cases with and without an APOE  $\epsilon 4$  allele. Using two-way analysis of variance, the degree of rabaptin-5 immunoreactivity was not significantly different between the APOE  $\epsilon 4$  and non- $\epsilon 4$  groups ( $P = 0.62$ ) but was significantly greater in the global ischemia group than the control group ( $P < 0.001$ ). There was no significant interaction between the presence of an  $\epsilon 4$  allele and global ischemia ( $P = 0.98$ ). Histograms are mean  $\pm$  SEM. Average calculated from sum of CA1 + CA2 + CA3/4 + dentate gyrus + neocortex + 5.

the frequency of the  $\epsilon 2$  allele was too low to determine any associations this allele may have (two controls and three global ischemia cases had one  $\epsilon 2$  allele; there were no control or global ischemia  $\epsilon 2$  homozygotes). An average degree of neuronal rabaptin-5 and rab4 immunoreactivity was calculated based on the levels assessed in hippocampal sectors CA1, CA2, CA3/4, dentate fascia, and neocortex. Two-way analysis of variance showed that the degree of rabaptin-5 immunoreactivity was not significantly different between the APOE  $\epsilon 4$  and non- $\epsilon 4$  allele groups ( $P = 0.62$ ) but was significantly greater in the global ischemia group than the control group ( $P < 0.001$ ). There was no significant interaction between presence of the  $\epsilon 4$  allele and global ischemia ( $P = 0.98$ ). Two-way analysis of variance revealed that the degree of rab4 immunoreactivity was not significantly different between the APOE  $\epsilon 4$  and non- $\epsilon 4$  groups ( $P = 0.62$ ) and was not significantly different between the control and global ischemia groups ( $P = 0.10$ ). However, there was a



**Figure 6.** The average degree of neuronal rab4 immunoreactivity in temporal lobe in cases with and without an APOE  $\epsilon 4$  allele. Using two-way analysis of variance, the degree of rab4 immunoreactivity was not significantly different between the APOE  $\epsilon 4$  and non- $\epsilon 4$  groups ( $P = 0.62$ ) and was not significantly different between the control and global ischemia groups ( $P = 0.10$ ). There was a significant interaction between the presence of an  $\epsilon 4$  allele and global ischemia ( $P = 0.017$ ). Histograms are mean  $\pm$  SEM. Average calculated from sum of CA1 + CA2 + CA3/4 + dentate gyrus + neocortex + 5.

significant interaction between presence of an  $\epsilon 4$  allele and an episode of global ischemia ( $P = 0.017$ ). The mean increase in rab4 immunoreactivity after global ischemia was 0.9 units lower on the semiquantitative score in patients with  $\epsilon 4$  than in non- $\epsilon 4$  patients (with a 95% confidence interval of 0.2 to 1.6 units lower).

### *A $\beta$ Deposition and Neurofibrillary Tangles*

In all cases, the presence of A $\beta$  deposits and neurofibrillary tangles was determined using sections immunostained with anti-amyloid and anti-tau antibodies. A minority of controls and global ischemia patients displayed A $\beta$  deposits and neurofibrillary tangles. Four controls had amyloid deposits alone and one control displayed both amyloid deposits and neurofibrillary tangles. In the global ischemia group, five cases had amyloid deposits and two had both amyloid deposits and tangles. There was no association between *APOE* genotype and the presence of amyloid deposits or tangles in both controls and global ischemia individuals. Furthermore, the presence of amyloid deposits or tangles was not associated with increased levels of rabaptin-5 or rab4 immunoreactivity.

### *Discussion*

This is the first demonstration of alterations in the endocytic pathway of neurons and glia after global ischemia in humans. We have shown that rabaptin-5 and rab4 immunoreactivity is increased in neurons and to a limited extent in glia in the hippocampus and neocortex of medial temporal lobe after global ischemia compared to normal controls. Rabaptin-5 and rab4 are markers of endocytic pathway activity and therefore, these results indicate an up-regulation of the intracellular trafficking process in neurons and glia of brains subject to an episode of global ischemia. Furthermore, we report an *APOE* genotype difference in endocytic pathway alterations after global ischemia.

The patients in this study experienced an episode of global ischemia caused by cardiac arrest with subsequent cerebral reperfusion. It should be noted that controls might have some extent of terminal hypoxia and/or hypotension because most die from a cardiac arrest but this would also be true of the global ischemia cases. However, in controls, reperfusion is not occurring. Furthermore, in a previous study using this cohort of individuals, there was minimal evidence of ischemic neuronal damage in control patients.<sup>23</sup> In humans, an episode of global ischemia with subsequent reperfusion results in a relatively stereotyped pathology in which there is selective neuronal damage in the hippocampus and neocortex.<sup>24</sup> In AD, in which neuronal injury is also a predominant feature, alterations in the neuronal endocytic pathway have previously been reported.<sup>12</sup> This suggests that the cascade of events after neuronal damage may include changes in the trafficking of molecules from the plasma membrane through the endocytic system. In cortical pyramidal neurons from AD brain, early endosomal volume was significantly increased in neurons labeled

with antibodies to rab5 and rabaptin-5. In addition, elevated rab4 immunoreactivity was observed in pyramidal neurons in the frontal cortex of AD brain. These observations are believed to indicate an increase in endocytic pathway activity because overexpression of rab 5, rabaptin-5, and rab4 have all been shown to promote increased endocytosis.<sup>8,25–27</sup> In the present study, neuronal rabaptin-5 and rab4 immunoreactivity was markedly increased in all regions examined in the temporal lobe of patients after global ischemia. This suggests that neuronal damage caused by an episode of global ischemia may be precipitating these increases in endocytic activity. However, minimal rabaptin-5 and rab4 immunoreactivity was also detected in neurons from control brain. Furthermore, there was no association between ischemic neuronal damage and the degree of either neuronal rabaptin-5 or rab4 immunoreactivity. A possible explanation for these observations is that endocytic alterations appear at a very early stage of neuronal damage and therefore may be detected before there is morphological evidence of ischemic neurons. Endosomal alterations are present in cortical pyramidal neurons of young patients with Down syndrome,<sup>12</sup> before the development of neurofibrillary tangles and amyloid deposits and the onset of neurodegeneration. It is conceivable, therefore, that endocytic pathway alterations are a very early response before neuronal damage can be visualized histologically.

The results from this study suggest that overall endocytic pathway activity is increased after global ischemia. Furthermore, the two distinct stages of endocytosis labeled by antibodies to rabaptin-5 and rab4, enable separation of the responses of these two phases of endosomal trafficking. Rabaptin-5 localizes to early endosomes and therefore an increase in rabaptin-5 immunoreactivity in neurons is indicative of an increase in the internalization and trafficking of material from the neuronal plasma membrane to early endosomes. In contrast, rab4 localizes to recycling endosomes that are returning to the plasma membrane and, consequently, an increase in immunostaining with rab4 antibody suggests an increase in transport of material back to the plasma membrane. Therefore, there is evidence of a parallel response of the internalization and recycling stages of endocytosis in neurons after global ischemia. This is supported by our finding of an association between neuronal rabaptin-5 and rab4 immunoreactivity after global ischemia ( $r^2 = 0.24$ ,  $P = 0.003$ ).

When patients were separated into groups based on *APOE* genotype, there were two notable observations. First, we determined that there was a statistically significant influence of *APOE* genotype on the degree of rab4 immunoreactivity changes in response to global ischemia. In contrast, there was no *APOE* genotype influence on the degree of rabaptin-5 immunoreactivity changes in response to global ischemia. This indicates that there is a differential response of the early trafficking and recycling stages of endocytosis after global ischemia in *APOE*  $\epsilon 4$  and non- $\epsilon 4$  individuals. Second, the data indicate a trend toward an increase in neuronal rab4 immunoreactivity in the *APOE*  $\epsilon 4$  control group, suggestive of underlying differences in endocytic pathway activity in control brain.

Up-regulation of recycling activity as a consequence of early AD is unlikely, because we found no evidence that the presence of amyloid deposits and/or neurofibrillary tangles influenced the extent of endosomal recycling. In individuals with amyloid deposits and/or tangles, we found no association with *APOE* genotype or levels of immunoreactivity of our endocytic markers. Higher levels of rab4 immunoreactivity in the control  $\epsilon 4$  group may reflect *APOE* isoform-specific intrinsic differences in endosomal trafficking. In previous studies, *APOE* genotype has been shown to influence other aspects of general brain function in uninjured brain, such as cerebral glucose metabolism.<sup>28</sup> Cell culture studies have demonstrated isoform-specific differences in cellular trafficking and localization of apoE in uninjured neurons. DeKroon and Armati<sup>29</sup> have shown that cultured human brain neurons incubated with apoE E3 or E4 traffic each isoform in a different manner. ApoE E3 and E4 have been demonstrated to accumulate in different parts of the neuron and show variation in interaction with cellular proteins.<sup>30,31</sup> Isoform-specific effects influencing cellular physiology in uninjured brain may therefore underlie the apparent discrepancy in endocytic recycling activity between the two control groups, an important finding in itself. It should also be noted that the *APOE* isoform-specific effects described above, in both controls and global ischemia cases, are unlikely to simply be a reflection of differences in the degree of neuronal damage. In a previous study using this cohort of patients, possession of an *APOE*  $\epsilon 4$  allele did not influence the degree of neuronal damage after global ischemia or in controls.<sup>23</sup>

It is possible that an *APOE* genotype influence on outcome after brain injury is mediated through endocytic pathway interactions. Neurons use endocytosis to take up many important macromolecules from the extracellular environment, including lipids and cholesterol. Lipids are particularly important building blocks of neuronal plasma membranes and myelin sheaths of axons. In the central nervous system, apoE acts as a lipid transport protein to deliver bound lipids to cells.<sup>32,33</sup> Sites in the amino-terminal domain interact with plasma membrane receptors, such as the low-density lipoprotein receptor-related protein (LRP), to facilitate internalization of the apoE-lipid-receptor complex. After neuronal damage, efficient delivery and uptake of lipid molecules is likely to enhance neuronal repair and survival<sup>34</sup> and this requires a maximally operational endocytic pathway. Our data demonstrate a differential response of the endocytic pathway to global ischemia in  $\epsilon 4$  and non- $\epsilon 4$  individuals. In  $\epsilon 4$  individuals, there is a trend toward a reduced number of endosomes being recycled back to the plasma membrane after global ischemia, compared to non- $\epsilon 4$  individuals in which there is a trend toward increased activity. An increase in the internalization and trafficking of molecules to early endosomes would facilitate increased uptake of lipids into the neuronal cytoplasm where they can be directed for membrane integration. After early endosomes have released cargo destined for other sites in the neuron, such as lipids, they can return to the plasma membrane and in turn recycle apoE and LRP for subsequent reuse. If there are fewer endosomes be-

ing recycled, there will be a resultant reduction in the amount of apoE and LRP recycled that may result in impaired uptake of lipids. Consequently, membrane reorganization and repair may be less effective in *APOE*  $\epsilon 4$  patients, which is likely to have a major influence on outcome to injury.

Membrane rebuilding is an integral part of brain plasticity requiring both adequate supply and uptake of lipids. We have described how lipid uptake may be compromised in  $\epsilon 4$  individuals after global ischemia. The role of apoE in the scavenging and delivery of lipids to neurons has previously been shown to be an integral component of the neuronal repair process.<sup>35,36</sup> ApoE is thought to scavenge and transport large quantities of lipids and cholesterol released from degenerating axon membranes and myelin<sup>37,38</sup> and deliver these to damaged but surviving neurons for repair. *In vitro* studies have demonstrated that the apoE E3 isoform is more effective in promoting neuronal survival and neurite outgrowth than apoE E4.<sup>30,39</sup> It is possible, therefore, that individuals with an *APOE*  $\epsilon 4$  allele will have impaired delivery of lipids and cholesterol to neurons in addition to a compromised ability to internalize these molecules after brain injury.

Findings from *in vitro* studies investigating the transport of apoE isoforms through the endocytic pathway have further emphasized the significance of apoE isoform-specific variations in endocytic behavior. Using marker molecules for different compartments of the endocytic system, progression of apoE E3 and apoE E4 through these compartments was shown to be different.<sup>29</sup> Although both isoforms were trafficked similarly through early endosomes, subsequently, apoE E4 was localized to late endosomes and lysosomes, whereas there was little evidence of apoE E3 after this route. This is intriguing as it suggests that apoE E3 may follow a recycling pathway back to the plasma membrane along with its associated low-density LRP. Such a route would enhance the lipid delivery system during neuronal repair and is consistent with our finding that endosomal recycling is increased in *APOE*  $\epsilon 3$  patients after global ischemia.

Thus far, we have proposed that endocytic pathway alterations may be involved in repair processes after brain injury. It is also possible these changes are involved in neuronal death/apoptotic pathways, although our finding of a lack of association of rabaptin-5 and rab4 staining with the extent of neuronal damage would argue against this. However, this cannot exclude the possibility that endosomal alterations are an early response before neuropathological evidence of neuronal death appears, as we discussed above. Previously, after neuronal injury, the lysosomal system was shown to be up-regulated before the appearance of histological and biochemical markers of neuronal death.<sup>40</sup>

The results from this study may also be of relevance to AD. The endocytic pathway is a key point of convergence for several molecules implicated in AD pathogenesis, such as amyloid precursor protein,  $A\beta$ , apoE, and the LRP.<sup>12</sup> As already discussed, endosomal alterations in neurons of young patients with Down syndrome have been observed. It is possible that alterations in the endo-



cytic pathway contribute to changes in neuronal processes years before the onset of clinical signs of AD. Such alterations could affect the normal interactions and trafficking of molecules such as apoE, LRP, and amyloid precursor protein with detrimental consequences on neuronal function. Furthermore, a genetic influence mediated by *APOE* polymorphism may underlie changes observed in endocytosis. Our results would suggest that possession of the  $\epsilon 4$  allele is associated with a reduced capacity for endocytic adaptations that promote neuronal repair. It has been proposed that individuals with an *APOE*  $\epsilon 4$  allele have a reduced ability to compensate for age-related neuronal loss<sup>41</sup> and in patients with AD, plastic neuronal remodeling was shown to be impaired in individuals carrying an  $\epsilon 4$  allele.<sup>42</sup> *APOE* genotype-specific effects on endocytic function may be involved in processes underlying these observations.

This study highlights a role for the endocytic system in the response to brain injury. However, there is a need for further investigation of how the pathway functions in both normal and disease states. Additionally, elucidation of the interactions between endocytosis and molecules implicated in acute brain injury and AD pathophysiology, such as apoE and  $\alpha\beta$  are required.

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